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Modification of Ontogenic Expression Due to Relocation of the ry+ Gene in Drosophila melanogaster

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MODIFICATION OF ONTOGENIC EXPRESSION DUE TO RELOCATION OF THE ry^+ GENE IN *DROSOPHILA MELANOGASTER*

by

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Approved:

Major Professor	Committee Member
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Allen A. Badgett Jr.

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ABSTRACT

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of the *ry*$^{+}$ Gene in *Drosophila melanogaster*

by

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Utah State University, 1973

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The *ry*$^{+}$ gene of *Drosophila melanogaster* and its associated enzyme, xanthine dehydrogenase, were employed in an analysis of the relationship between regulation and the location of a gene in the genome. Enzyme assays as an indication of gene activity were performed on genotypes containing zero, one and two doses of *ry*$^{+}$ genes. Xanthine dehydrogenase activity of rosy genes in normal and relocated positions was determined during development.

The results indicate: 1) the gene *ry*$^{+}$ is differentially active during ontogeny; 2) the activity during ontogeny is proportional to the number of *ry*$^{+}$ genes present in the genome; and 3) a unique level of xanthine dehydrogenase activity is associated with relocated *ry*$^{+}$ genes in the absence of a structurally normal third chromosome.

Genotypes containing relocated *ry*$^{+}$ genes have a similar developmental curve of enzyme activity as that of the control. The genotypes with a relocated

$\underline{ry}^+$ gene and lacking a structurally normal third chromosome consistently have a higher level of enzyme activity than the control, except in the late pupal stage. This increased level of activity is not the result of a general physiological effect since the ontogenic profile of glucose-6-phosphate dehydrogenase activity is the same in wild type and relocated $\underline{ry}^+$ genotypes. The relevance of the data to models for gene regulation is discussed.

(75 pages)

INTRODUCTION

An important aspect of growth and development involves the temporal regulation of protein synthesis. The mechanisms involved in gene regulation in higher organisms are, for the most part, not known. Any proposed mechanism must explain how genetically identical cells become morphologically and functionally distinct. It must also explain how differentiated cells are coordinated into a functional unit.

The fact that gene expression is affected by the position of the gene within the genome is shown by the phenomena of variegated position effect (see Baker, 1968 for review). Study of this effect has made it clear that there is a modification of gene expression due to relocation of the gene adjacent to a break in heterochromatin.

It is also known that a break in the vicinity of, but not at, the gene locus can result in mutant expression. Muller (1962, p. 14) has shown that the phenotype produced by the scute locus is varied when rearrangements occur directly adjacent to the gene. Tobler (1971) has shown that the tryptophan pyrrolase activity specified by the v^+ locus in Drosophila melanogaster is lower in a genotype containing a relocated vermilion gene than in control Canton-s. Bowman and Simmons (In press) have reported that the 6-phosphogluconate dehydrogenase (6PGD) activity associated with the Pgd^+ gene of Drosophila melanogaster is

significantly lower in translocated males than in structurally normal males.

The above evidence leads to the hypothesis that the structure of the genome is an intrinsic part of regulation. Therefore, a gene in a different location in the genome would be subject to different regulatory programs.

This type of hypothesis for regulation is a simple one and is consistent with recent proposals for gene regulation in higher organisms (e.g. Britten and Davidson, 1969).

The object of this investigation was to test the hypothesis that the structure of the genome is an intrinsic part of regulation. This was done by determining if relocating a gene by translocation affects the expression of that gene at any particular stage of development.

The rosy gene of Drosophila melanogaster and its associated enzyme activity xanthine dehydrogenase (XDH), were employed in an analysis of the relationship between regulation and the location of genes in the genome. Enzyme assays as an indication of gene activity, were performed on genotypes containing zero, one and two doses of ry^+ in normal and relocated positions in Drosophila melanogaster. The ontogenic profile of xanthine dehydrogenase activity of relocated and normally positioned rosy genes was determined.

Relocating a gene may cause a change in the enzyme associated with the relocated gene. To test this possibility, electrophoretic mobility and kinetic parameters of xanthine dehydrogenase produced by relocated rosy genes were determined and compared with xanthine dehydrogenase produced by the control Canton-s.

To insure that any difference detected in genotypes containing a relocated rosy gene was not due to a general physiological variation in strains, glucose 6-phosphate dehydrogenase activity was determined concomitant with all XDH assays.

REVIEW OF LITERATURE

Ontogenic Regulation

The ontogenic expression of various enzyme activities presents an ideal method of determining the chromosomal parameters affecting ordered activation and repression of gene action. Many enzymes are associated with specific cell types and particular stages of differentiation (e.g., Rutter, Kemp, Brodshaw, Clark, Ronzio and Saunders, 1968; Wright and Shaw, 1970). The differentiation of cellular types and the determination of their functions is due to a program of ordered activation and repression of gene action. Enzyme activity is presently the best indication of gene activity during development.

There is a temporal regulation of protein synthesis during the development of higher organisms. A number of examples of this temporal regulation and the use of enzyme activities to measure it will follow. Courtright (1967) using electrophoresis and biochemical assays studied the ontogenic expression of aldehyde oxidase in D. melanogaster and D. similis. He reported a low level of activity at all stages earlier than the third larval instar, followed by a steady increase in activity. Ursprung, Smith, Sofer and Sullivan (1968) reported on the ontogenic expression of alcohol dehydrogenase (ADH) and aldehyde oxidase in D. melanogaster and showed that the two profiles were different. Dunn, Wilson and Jacobson (1960) showed that with the change in ADH activity during ontogeny there is also a change in relative concentrations of ADH isozymes.

Whitney and Lucchesi (1972) reported studying fumarase activity during the development of D. melanogaster in both males and females. Profiles were found to be quite similar in males and females during larval and pupal stages, but differed markedly in post-emergence imagoes. They attribute this to a greater increase of soluble protein in the female, probably due to egg production, and a resultant drop in specific activity relative to protein. Male maturation does not involve such a great increase in soluble protein. Consequently, male fumarase specific activity increases until it is greater than female activity two days after emergence. A similar effect has been shown for G6PD in Drosophila by Steele, Young and Childs (1969).

Catheptic and acid phosphatase activity have been studied in developing D. melanogaster (Mulherkar, Kothari, and Vaidga, 1972). Catheptic activity was found to be absent in the egg, in first, and in second larval instars. Activity first appears in the third instar larvae and the activity peaks 24 to 48 hours after puparium formation. It then decreases, at first gradually, reaching zero just before emergence of the imago. The profile of activity for acid phosphatase was reported as being similar to catheptic activity in the larval and pupal stages. Acid phosphatase activity was found to be exceptionally high in the egg, in contrast to catheptic activity. Friedman (1973) reported detecting uricase activity in only the larvae and imagoes. No uricase activity was found in the pupae. He also reported that ma-1 and ry^2 males and females had 10-30 times the activity detected in wild type (Ore-R, Swedish-b and Canton-s).

Britten and Davidson (1969) have proposed a theory for the "genomic regulation" of higher organisms. They propose that batteries of producer genes are regulated by activator RNA molecules synthesized on intergrator genes. The effect of the intergrator gene is to induce transcription of many producer genes in response to a single molecular event. Their theory is consistent with the idea that there is a temporal regulation of protein synthesis during development.

From the above discussion, it is clear that there is a temporal regulation of protein synthesis during development in Drosophila. It is also clear that enzyme activities can be used to reflect gene regulation during development. Specifically, enzyme activities can be used to determine if relocating a gene to a different part of the genome affects the gene's regulatory program.

Rosy and Xanthine Dehydrogenase

The objectives of this investigation make the following demands on the gene-enzyme system used: 1) the cytological location of the gene must be known; 2) the gene must be associated with an enzyme; 3) the same gene must be available in two or more chromosomal locations; 4) the effect of zero, one or two doses of the gene on enzyme activity must be known; and 5) a reliable assay system for the enzyme must be available.

The rosy-xanthine dehydrogenase system in Drosophila provides an excellent system to study the properties of regulation since it fulfills all of the above criteria. Rosy is an autosomal gene located on chromosome 3 at 52.0

(Lindsley and Grell, 1968). It has been mapped cytologically by G. Lefevre Jr. (1971) to the region 87D8 to 87D12. The rosy mutant has a reddish-brown eye color. The mutant lacks detectable amounts of the enzyme xanthine dehydrogenase. Several Drosophila stocks are available containing structural rearrangements of the third chromosome. Some of these rearrangements, involving euchromatin as well as heterochromatin, are due to breaks in the chromosome near the rosy locus.

Grell (1962) studied the dosage effect of ry^+ on xanthine dehydrogenase activity in Drosophila melanogaster. Comparisons of XDH activities in flies with one, two or three doses of ry^+ revealed an increase in enzyme activity proportional to dose in both males and females. A number of investigators have examined xanthine dehydrogenase activity during development at 25 C in wild type flies (Glassman and McLean, 1962; Munz, 1964). Significant activity has been detected as early as the first larval instar. A sensitive fluorometric assay for XDH activity in Drosophila extracts has been devised by Glassman (1962). The assay is reliable and easily performed on even a single fly.

Genetic analysis of the rosy region of chromosome 3 has shown that there is only one area of the third chromosome concerned with XDH production (Schalet, Kernoghan and Chovnick, 1964). They have placed precise limits on the extent of the rosy unit (Figure 1). To the left of rosy is the locus of messy (mes). Analysis shows that mes mutants lie no further than 5×10^{-3} crossover units from the rosy region. Located to the right of rosy are piccolo (pic), A, B and

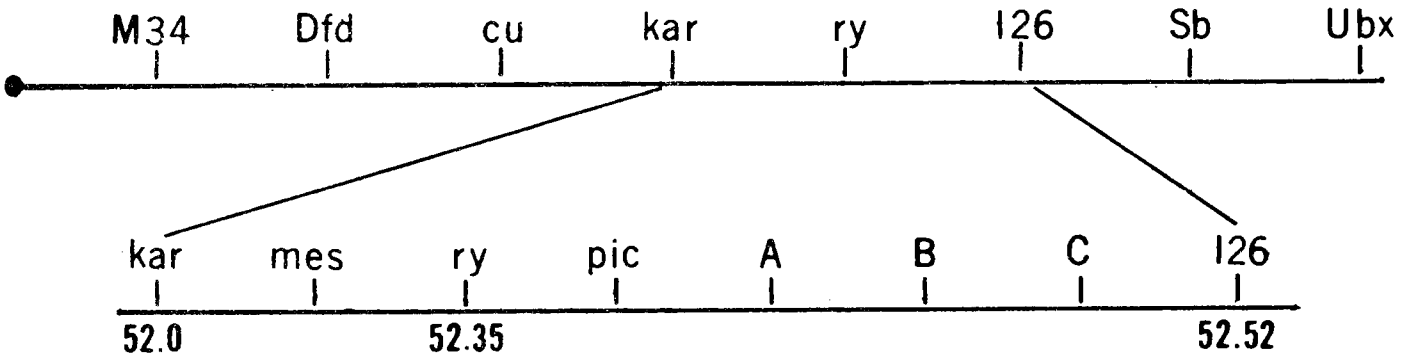


Figure 1. Linkage relations in the rosy region of Chromosome 3.

C, which are postulated genetic units of unknown effect (Chovnick, 1966) and lethal-26 (1-26).

In an effort to determine if two or more cistrons could be found in the rosy region of chromosome 3, Chovnick (1966) studied 24 rosy-like X-ray-induced mutants. All mutants isolated were found to be members of the single rosy cistron. They also tested the possibility that there might be non-rosy-like mutants near the rosy region that affect XDH activity. The non-rosy-like mutants tested, failed to show a functional relationship with rosy.

Chovnick (1966) has used the rosy locus to demonstrate that intracistronic recombination does occur in Drosophila and that it is possible to identify genetic units of function. These units consist of a linear order of sites subject to mutation.

From his work on the XDH mutants, Chovnick (1966) has concluded: 1) there is only one cistron affecting XDH in the region tested on chromosome 3; 2) mutations within this cistron are viable; 3) no complementation has been shown in the rosy cistron; and 4) there are functionally distinct units of genetic activity adjacent to the rosy cistron.

Although not directly relevant to the relationship between rosy and XDH, it is important to note that the production of XDH is also influenced by low xanthine dehydrogenase (lxd) at 33 $\pm$ on the third chromosome (Keller and Glassman, 1964a, 1964b); and maroon-like eye color (ma-1) at 64 $\pm$ on the X chromosome (Glassman and Mitchell, 1959a; Hubby and Forrest, 1960).

Hadorn and Schwinck (1956) transplanted tissue from wild type flies into ma-1 and ry larvae, resulting in flies with wild type eye color. They were unable to isolate the active substance, since extracts of wild type larvae, pupae or adults fail to produce any effect when injected into ry or ma-1 larvae. Glassman (1965) offers a possible explanation, "that the active substance is autoxidizable and thus inactivated during extraction and injection." He has reported studying gynandromorphs which are heterozygous (ma-1/+) on the right side and hemizygous (ma-1) on the left side. Even though the left side should not have detectable levels of XDH, since it lacks a $ma\text{-}1^{+}$, the left eye is phenotypically wild type. Therefore, he concludes that there is a diffusible substance. Attempts to determine what the diffusible substance is have failed (Glassman, 1965).

Extracts of ma-1, $ma\text{-}1^{bz}$, $ma\text{-}1^{2}$ and lxd have been shown to contain a cross-reacting material (CRM) which precipitates antibodies formed in response to Drosophila XDH (Glassman and Mitchell, 1959b; Glassman, 1965). Keller and Glassman (1965) report that ry^{+} is essential for the production of CRM.

Mixtures of extracts of ma-1 and ry mutants, result in XDH activity (Glassman, 1962b). This complementation has been demonstrated using extracts from a number of different ma-1 alleles incubated with extracts of the mutants ry^{1} and ry^{2} (Glassman, 1965). The ry^{+} complementing factor is inactivated by trypsin and chymotrypsin, is precipitated by ammonium sulfate, and is sensitive to heat treatment. Therefore, it seems highly probable that the ry^{+} complementing factor is a protein. The $ma\text{-}1^{+}$ complementing factor also has many characteristic pro-

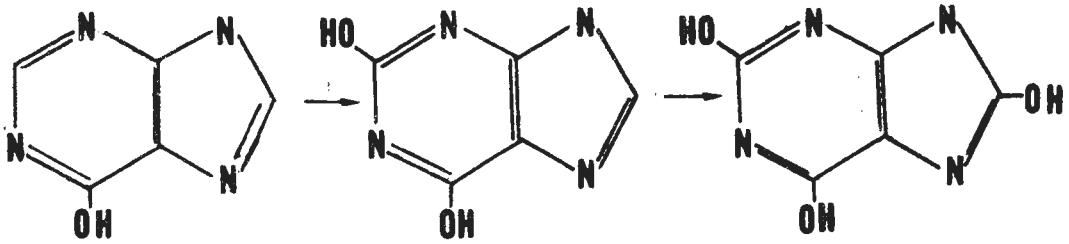
tein traits. Neither ma-1^{+} nor ry^{+} complementing factors have been isolated (Glassman, 1962b).

Glassman and Pinkerton (1960) reported intragenic complementation in a female heterozygous for the two alleles ma-1 and ma-1^{bz}. These females have wild type eye color and 5 to 10 percent of normal XDH activity found in wild type flies. They have failed in attempts to demonstrate in vitro complementation between extracts of ma-1 and ma-1^{bz}. They also reported that the allele ma-1^{2} fails to show complementation in vivo with either ma-1 or ma-1^{bz}. Although the in vivo complementation results in normal eye color, the low level of XDH present is reflected by the small amounts of reaction products (uric acid and isoxanthopterin) and the accumulation of substrates (hypoxanthine and 2-amino-4-hydroxypterine).

Parzen and Fox (1964) reported a 530 fold purification of XDH. They also report an assay in which XDH activity is determined by measuring the change in absorbence at 340 mμ as nicotinamide-adenine dinucleotide (NAD) is reduced in the presence of substrate xanthine or hypoxanthine (Figure 2). Tests of assay reliability were performed on one-day-old single flies, male and female, from both inbred and non-inbred stocks. No significant difference was reported between males and females, but a significant difference was reported between different strains. Whether these differences are real or a product of differences in the size of flies of different stocks is unclear. To test this difference, it would be necessary to use a common denominator between stocks, such as protein levels. This

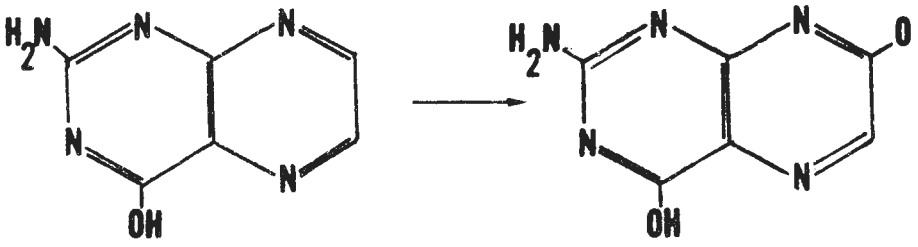
Figure 2. Reactions catalyzed by xanthine dehydrogenase in Drosophila.

PURINES

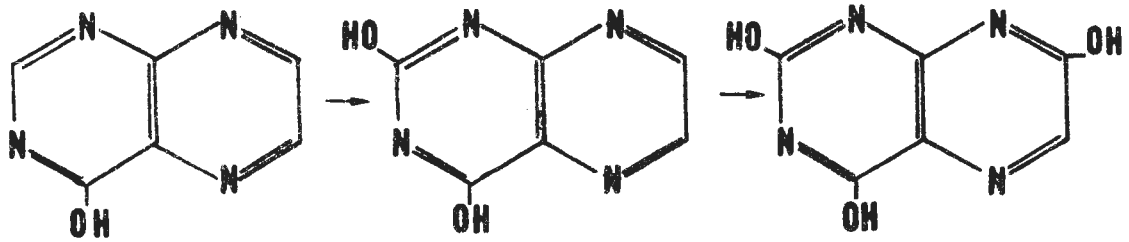


HYPOXANTHINE XANTHINE URIC ACID

PTERIDINES



2-AMINO-4-HYDROXY PTERIDINE ISOXANTHOPTERIN



4-HYDROXYPTERIDINE 2,4 DIHYDROXY PTERIDINE 2,4,7 TRIHYDROXY PTERIDINE

is particularly evident since Glassman (1962b) reports a difference between males and females in XDH activity.

Four alleles of ry^+ have been identified by electrophoresis (Yen and Glassman, 1965). These alleles have been called ry^{el-S}, ry^{el-SI}, ry^{el-I} and ry^{el-F}, where el stands for electrophoretic variant, and S, SI, I, and F stand for slow, slow intermediate, intermediate and fast mobilities. Yen and Glassman (1965) found these four electrophoretic variants of XDH among 56 strains of Drosophila melanogaster. They found that when flies from different strains containing similar variants of XDH were crossed, the XDH produced by the progeny did not differ with respect to mobility. Crosses involving parents with varient XDH mobilities produced progeny in which the XDH electrophoretic mobility was intermediate between those of the parents. Crosses of the four variants with ma-1, ry and lxd indicated that the ry^+ locus is probably responsible for determining electrophoretic mobility. Yen and Glassman carried out an extensive genetic analysis to confirm that the electrophoretic variants were determined by the ry^+ locus. They were able to confirm that the gene determining mobility of XDH variants is located midway between Wrinkled (W, 3-46.0) and Stubble (Sb, 3-58.2). This is consistent with ry^+ being the determining locus for electrophoretic mobility. They feel that this does not rule cut the possibility that a locus closely linked to ry^+ is responsible. They also suggest that their data does not exclude the possibility that ry mutants may have a defective operator-like gene which controls activity of the adjacent structural gene for XDH. Their work rules out the possibility that $ma\text{-}1^+$ or lxd^+ are loci that

determine electrophoretic mobility.

Using xanthine as a substrate, the Km of XDH has been reported as 25 μM by Glassman and Mitchell (1959a), 23.6 μM by Parzen and Fox (1964) and 21 μM by Yen and Glassman (1967). Using AHP as a substrate, the Km of XDH has been reported as 6.7 μM at pH 8.0 (Glassman and Mitchell, 1959a; Yen and Glassman, 1967).

Using 2-amino-4-hydroxypteridine-6-carboxyaldehyde (a pteridine), 8-azaguanine (a purine) and ammeline (a triazine), inhibition studies were performed by Yen and Glassman (1967). When both substrate and inhibitor are either pteridines or purines the inhibition is competitive, indicating that inhibitor and substrate are competing for the same site. When a purine is the substrate and pteridine is the inhibitor, or the reverse, the inhibition is noncompetitive. This indicates the presence of a site for purines, and one for pteridines on the enzyme. Yen and Glassman (1967) studied the kinetic properties of four types of electrophoretic variants of XDH. They indicate no significant differences in the kinetic parameters studied among these electrophoretic variants. Therefore, it appears that the alteration of amino acid composition in xanthine dehydrogenase, as reflected by electrophoretic mobilities, does not change the enzyme so as to affect its catalytic functions.

It is clear from the above discussion, that the rosy-xanthine dehydrogenase system fills the requirements necessary for a study on the effect on regulation of relocating a gene. Rosy is the structural gene for xanthine dehydrogenase,

a non-essential enzyme. A number of mutants of rosy, including a null allele, and several rearrangements in which the rosy gene is relocated are available. The rosy gene displays dosage additivity as reflected by a proportional increase in XDH activity with increasing numbers of rosy genes. A reliable assay system for xanthine dehydrogenase activity has been developed.

Position Effect

The phenomenon of position effect has long been recognized as a fundamental problem in genetics. A number of advances have been made in the understanding of position effect in Drosophila, but the considerable body of evidence relating to this subject has yet to be incorporated into a logical and consistent theory. It is known that there are at least two distinct types of position effects, stable and variegated (Lewis, 1950). Stable (S-type) position effects are characterized by somatically stable phenotypes and involve wholly euchromatic regions of chromosomes. A considerable number of chromosomal rearrangements in Drosophila have been found to be associated with stable changes in gene action. Demerec (1940) and co-workers noted that in a number of cases the affected gene is usually adjacent to or only a few bands removed from the point of rearrangement. The variegated type of position effect (V-type) is characterized by somatically mosaic phenotypes. The phenomenon involves a relocation of a gene from a euchromatic region into the vicinity of heterochromatin.

A large number of genes in Drosophila are now known to exhibit variegated position effect when brought into association with heterochromatin. This phenomenon of variegated position effect does not appear to be restricted to any particular gene. Relatively few genes have effects which make them suitable for a study of the variegated phenotype. The most widely studied example in which variegation can be detected as a color change due to the presence of red and white facets in the compound eye.

In discussing variegated position effect, it is convenient to introduce a notation expressing the presence of a rearrangement (R) and the allele present at the time the rearrangement was produced. Stern and Heidenthal (1944) used this terminology for rearrangements of the cubitus interruptus (ci) gene on chromosome 4. Thus R($\underline{w}^+$) designates a rearrangement associated with the normal allele of the white gene. The R will denote a rearrangement involving euchromatin and heterochromatin.

Considerable work on the phenotypes associated with R($\underline{w}^+$) types has been reported by Muller (1930), Gowen and Gay (1934) and Demerec and Slizynska (1937). The variegated position effect is demonstrated by the fact that the heterozygous R($\underline{w}^+$)/$\underline{w}$ and viable R($\underline{w}^+$) homozygotes and hemizygotes have variegated eyes. The R($\underline{w}^+$)/$\underline{w}^+$ genotypes have normal red eyes. The variegation shows an extraordinary range of variability depending on the particular R($\underline{w}^+$) genotype being considered.

A number of cases are known in which variegation acts as a dominant character (Lewis, 1950). The R($\underline{bw}^+$)/$^\pm$ genotype shows marbled brown and red facets whereas the R($\underline{bw}^+$)/$\underline{bw}$ has an almost homogenous brown eye color (Glass, 1933).

Ephrussi and Sutton (1944) have interpreted dominance as an effect exerted by a rearrangement on the $\underline{bw}^+$ allele in the normal as well as in the rearranged chromosome. If this interpretation is correct, then R($\underline{bw}$)/$^\pm$ flies should show dominant brown-variegation. This was shown to be the case by Glass (1933).

Dubinin and Sidorov (1935), as cited by Lewis (1950), reported direct proof of the position effect phenomena using the hairy gene ($\underline{h}^+$) located on the left arm of chromosome 3. Using salivary gland chromosome analysis, they found a rearrangement in flies showing variegation for hairy. This rearrangement involved the proximal or heterochromatic region of chromosome 4 and the left arm of chromosome 3. By inserting first a mutant and then a normal $\underline{h}^+$ allele into the rearrangement, they showed that the introduced $\underline{h}^+$ allele acquired the same instability as the $\underline{h}^+$ allele in the original translocation. They concluded that the gene was not altered, but that the genes expression was changed by its new genetic environment.

Panshin (1935) using a translocation between the third and fourth chromosomes studied position effect on the curled gene ($\underline{cu}^+$). He reported five cases in which a mutant $\underline{cu}$ gene was inserted into a rearrangement. Into each of these five R($\underline{cu}$) genotypes a normal $\underline{cu}^+$ allele was then inserted yielding R($\underline{cu}^+$)

genotypes. Panshin (1935) showed that these five new R($\underline{cu}^{+}$) flies had the same variable phenotype as the original heterozygotes R($\underline{cu}^{+}$)/$\underline{cu}$. Therefore, the position effect was due to the rearrangement and not a change in the curled gene.

Baker (1968) suggests that position effect variegation operates at the transcriptional or translational level of gene expression. He mentions four facts to support this argument: 1) ubiquity of genes effected; 2) the $\underline{cis}$-$\underline{trans}$ nature of the effect; 3) the centromere distance effect; and 4) the polarized spreading effect. This fourth fact is most interesting. Suppression of gene activity spreads from the "heterochromatic" break point to the nearest gene and may effect several genes further removed from the break point. This suppressive action of the heterochromatic break has been shown to extend along the chromosome. Demerec (1940) has made extensive studies of this process. In the inversion N^{264-52} variegation of the expression of five genes was observed and correlated with the arrangement of these genes relative to the centromere region of the X chromosome. In this case, the effect was observed to spread as far as the $\underline{bifid}$ locus, located 50 to 65 salivary gland chromosome bands from the point of rearrangement. He also showed that this effect might extend up to 80 salivary gland chromosome bands from the point of rearrangement. In terms of recombination this represents a distance of about 6 centimorgans. Using the variegated peach gene in $\underline{D.\ virilis}$, Baker (1954) has shown that the spreading effect extends over one-third the length of chromosome 5. Russell and Montgomery (1965) in studies on the mouse reported that the spreading

effect may be observed 24 centimorgans from the break point.

Gowen and Gay (1933) reported that variegation was suppressed toward wild type in genotypes which included an extra Y chromosome. They also determined that the removal of a Y chromosome in a genotype showing variegation, increased variegation toward the mutant phenotype.

Suppression of variegation is associated with addition of heterochromatin and is not limited to heterochromatin of the Y chromosome. Noujdin (1944) reported that the addition of Y heterochromatin, and heterochromatin from the X and fourth chromosomes were effective in suppressing variegation.

Grell (1959a) reported a gynandromorph in D. melanogaster that was variegated for the brown locus. The somatic loss of an X chromosome on the left side enhanced the mutant expression, and, therefore, had the same effect as the loss of a Y.

Baker and Spofford (1959) investigated the effect of 15 different fragments of the Y chromosome on white variegation in Drosophila melanogaster. They found that Y fragments differed considerably in their suppressive ability. Some were more effective in suppressing the variegated position effect than complete Y chromosomes. This ability was not necessarily correlated with their cytological length.

Brosseau (1960) utilized a series of Y fragments to study the suppression of variegated position effect at the Bar locus. Brosseau (1964) was able to show the presence of one suppressor of variegation to be located near the kl-s fertility

factor in Y^L and the presence of another on Y^S proximal to ks-1. Baker (1968) concluded that there are discrete loci which suppress variegation located on the Y chromosome, and that most Y chromosomes contain alleles of these genes that are active.

One notable exception to the general rule of Y chromosome suppression has been reported by Schultz (1936). He found that the addition of a Y chromosome enhanced the variegated mutant tissue in the mutant light, whereas, the deletion of a Y suppressed the mutant tissue. Baker and Rein (1962) confirmed that this is the case and is not the result of experimental error. They introduced the same Y chromosome, or fragment, into single individual males showing variegation of light and also into genotypes which showed white variegation. The Y fragments that were the most effective in suppressing mutant tissue in white variegated genotypes were the most effective in enhancing mutant expression in the light system.

Lewis (1950) suggested that this enhancement of the variegated position effect by Y chromosomes is characteristic of genes located in the heterochromatic region. To determine if this is the case, Grell (1959b) studied cubitus interruptus, which is located in the heterochromatin of the fourth chromosome. He found that in R($\underline{ci}^+$)/$\underline{ci}$ heterozygous females the addition of a Y chromosome shortens the gap in the wing vein. The presence of two Y's made the vein more nearly normal that one Y. Schneider (1962) studied the peach gene in $\underline{D.\ virilis}$, a gene that is also located in the heterochromatic region. He found that an extra Y reduced the mutant tissue in the variegated eye.

Gowen and Gay (1933, 1934) have shown that high temperature tends to suppress variegation and low temperature enhances it. Chen (1948), using R($\underline{w}^+$) found that this temperature treatment is effective only if applied during development.

Schultz (1941) has reported several cases in which a rearrangement between euchromatic and heterochromatic regions acts as a modifier on variegation due to a different rearrangement.

Gersh and Ephrussi (1946) studied the influence of deficiencies near the white gene in R($\underline{w}^+$)/$\underline{w}$ heterozygotes. When allowance is made for loss of extreme variegated flies due to the presence of lethal genes, their results indicate that deficiencies enhance variegation.

In individuals homozygous for a rearrangement having a variegated position effect, it might be anticipated that less mutant tissue would be present than in individuals which are heterozygous for the rearrangement (Lewis, 1950). This should be true since the chance that an individual cell would have both normal alleles impaired in function due to the rearrangement, would depend on two separate events. However, Demerec and Slizynska (1937) reported that the R($\underline{w}^+$) homozygote have lighter eyes than the heterozygote in the case of $\underline{w}^{258-18}$. Schultz (1941) has remarked that, "homozygotes always show more variegation than the heterozygotes." Lewis (1950) suggests that perhaps somatic pairing in the homozygous rearrangement brings about a closer association of heterochromatin in the effected pair of alleles than is possible in the heterozygote.

Noujdin (1944) states that structural heterozygosity or homozygosity modifies not only the individual in which it occurs, but also exerts an influence on the offspring. Even though Noujdin (1946) was unable to duplicate his earlier work, other workers, notably Spofford (1959) and Hessler (1961) have shown that homozygosity for the rearrangement in the parent affected the heterozygous progeny studied. If the mother is homozygous for the rearrangement, there is more wild type tissue in the variegated phenotype of the offspring than if she is heterozygous. Schneider (1962) observed that some suppression of variegation is present when the father is homozygous for the rearrangement. She also showed that variegation of the peach gene in D. virilis is subject to suppression by the presence of a Y in the mother. Spofford (1959) reported a similar effect not only with extra Y's, but also with Y fragments. One of these Y fragments was even more effective than a complete Y in this maternal suppression.

Two possible models might be proposed to explain the phenomena associated with variegated position effect: 1) mutations, an actual change or alteration in the informational DNA; and 2) a change in control of gene activity. The first model does not agree well with available information associated with variegated position effect. For the first model to conform with available data, one must propose that moving a gene back to its original position reduces its mutability; that extra heterochromatin can act both as a suppressor and activator of mutations; and that decreases in temperature during development are mutagenic.

While all of these lines of evidence are difficult to reconcile with a mechanism of somatic mutation, they are consistent with a mechanism involving a differential change in gene activity within a tissue.

MATERIALS AND METHODS

Drosophila Stocks

The Drosophila stocks used in these experiments were obtained from the California Institute of Technology, Pasadena, California and from Dr. A. Chovnick, University of Connecticut, Storrs, Connecticut. All stocks used were crossed to the Canton-s wild type to reduce genetic variation. Pertinent information about stocks and mutants used is given in Table 1. Additional information is available in Lindsley and Grell (1967). All stocks were maintained at 25 C on standard cornmeal-sugar-yeast medium.

Collection Procedure

The experiments performed in this study required samples of developing flies at closely timed intervals. To insure synchrony, selected genotypes were placed in half-pint milk bottles containing 3 percent agar covered with moistened bakers yeast. The parents were maintained on freshly yeasted bottles for 12 hours to reduce egg retention by the females. The parents were then removed and placed on a second set of freshly yeasted bottles for 12 hours. The parents were then removed and the eggs incubated at 25 C until the desired developmental stage was reached. At 24 hour intervals the developing larvae were collected by

Table 1. Synopsis of mutants, chromosome aberrations and wild type stocks of Drosophila melanogaster used in this study. Additional data in Lindsley and Grell (1967)

Designation	Description
+	Canton-s wild type
ry	rosy-eye, 3-52.0, 87D8-12
Zw	Zwischenferment (glucose-6-phosphate dehydrogenase), X-63
$Df(3R)ry^{27}$	Deficient for rosy, homozygous lethal
$Df(3R)ry^{81}$	Deficient for rosy, break points at 87C2 and 87D14-E1
T(3;4)86D	Reciprocal translocation between the right arm of chromosome 3 and the basal portion of the right arm of chromosome 4, break points in chromosome 3 at 86D1-2 and in chromosome 4 at 101F (Grell, 1959a, 1959b)
$Dp(3;4)ry^{+}$	Derivative of the $3R^{D}4^{P}$ elements of T(3;4) 86D, carries normal alleles of cu, kar and ry

washing onto a fine mesh screen and assayed for xanthine dehydrogenase (XDH) and glucose 6-phosphate dehydrogenase (G6PD) activity.

Synchronization of pupal and imagonal stages was enhanced by washing the developing animals into large beakers of water (25 C) 96 hours after hatching. The floating pupae were discarded and the non-floaters were placed onto freshly yeasted bottles for 6 hours. They were again washed into large beakers of water and the floating pupae collected (Mitchell and Mitchell, 1965). This sample included all pupae that had undergone head eversion.

Collected floaters were placed on moistened filter paper in petri dishes at 25 C until selected for assay. Samples were selected at 24 hour intervals and assayed for XDH and G6PD activity.

Adults were collected at 12 $\pm$ 3 hours after eclosion and aged, at 25 C, until selected for assay. Samples were selected at 12 hour intervals for 96 hours and assayed for XDH and G6PD.

Enzyme Analysis

Xanthine dehydrogenase

The XDH assay used was a modification of that described by Glassman (1962a). Larvae (40), pupae (40) or imagoes (60) were hand homogenized in 2 ml of distilled water in a 2 ml Ten Broeck (Kontes) grinder. All steps during extraction and preparation were carried out at 0-5 C. One milliliter of the homogenate was diluted with 3 ml of 0.2 M phosphate buffer (pH 7.2) and then added to 10 mg

of charcoal (Norit A) and stirred. After 20 minutes, this mixture was centrifuged in a Sorvall RC-2 centrifuge for 20 minutes at 30,000 x g. The resulting supernatant was filtered through a Pasteur pipette containing glass wool. After filtering, 2 ml of the filtrate was added to 3 ml of 0.2 M phosphate buffer (pH 7.2) in a fluorometer cuvette and kept at 30 C in a Sero-Block (Model 13) dry bath. When the reaction mixture reached 30 C (approximately 5 minutes), 0.1 ml of 0.001 M methylene blue and 0.07 ml of 0.001 M 2-amino-4-hydroxypterine were added. The reaction mixture was stirred initially and readings taken at 5 minute intervals for 20 minutes in a Turner Fluorometer (Model 110) equipped with 360 mμ (Turner #110-811) and 350 mμ (Bauch and Lomb) primary filters and 405 mμ (Turner #110-812) and 10 percent Transmittance (Wratten N.D. 1.00) secondary filters. A standard curve relating isoxanthopterin to fluorometric units was determined. Enzyme activities were reported as nm isoxanthopterin produced/mg protein/minute.

The remaining 1 ml of homogenate was centrifuged in a Sorvall RC-2 for 20 minutes at 30,000 x g and used for determination of protein concentrations and G6PD assay. The method of Lowry (see Layne, 1963) was used to determine protein concentrations. Standard protein samples were tested with all experimental samples.

Standard biochemical techniques, such as determination of substrate saturation, pH and temperature sensitivity were carried out to validate the assay technique. Determined optimum conditions were maintained for all experiments.

Canton-s, as a control, was assayed for XDH and G6PD activities concomitantly with all experimental genotypes.

Glucose 6-phosphate dehydrogenase

The assay conditions for G6PD were those developed by Bowman and Simmons (1973, in press) from Seecof, Kaplan and Futch (1969). G6PD activity was determined by pipetting 0.2 ml of the supernatant into a spectrophotometer cuvette containing 1.8 ml of reaction mixture, giving a total volume of 2 ml. This 2 ml reaction mixture consisted of 1.5×10^{-3} M glucose-6-phosphate monosodium salt, 0.35×10^{-3} M nicotine-adenine-dinucleotide-phosphate monosodium salt, 3×10^{-3} M $MgCl_2$, and .15 M Tris HCl buffer (pH 7.5), plus homogenate. The change in optical density at 340 mμ was measured relative to a blank reaction mixture lacking glucose-6-phosphate. The assay was performed at 20 C in a Gilford (Model 222A) spectrophotometer with a recorder (Model 6040). Enzyme activities were expressed as nm NADP reduced/mg protein/minute. It was assumed that 1 mole of substrate was used for each mole of NADP reduced.

Temperature Inactivation Curve

A temperature inactivation curve of XDH activity was determined for three genotypes T(3;4)86D, Df(3)$\underline{ry}^{81}$, Df(3)$\underline{ry}^{81}$/Dp(3;4)$\underline{ry}^{+}$ and Canton-s. Homogenates of imagoes (24 $\pm$ 3 hours) were prepared as usual. After filtration through

glass wool, samples were incubated for 5 minutes in a Metabolyte Water Bath Shaker (New Brunswick, Model G77) at temperatures ranging from 20 to 80 C (5 C intervals). After incubation, XDH activity was determined by the usual techniques.

Electrophoresis

Disc electrophoresis on 5 percent cyanogum No. 41 polyacrylamide gel (American Cyanamide) followed a modification of Yen and Glassman (1965). The gel was made using 0.1 M Tris (hydroxymethyl) aminomethane (Sigma) at pH 9.0, 5.0 percent cyanogum, 0.1 percent tetramethylethylenediamine and 0.1 percent ammonium persulfate. The cyanogum solution was poured into glass tubes sealed at one end and allowed to polymerize for 90 minutes. The top of the gel was layered with brom-phenol blue which acted as a tracking dye. Excess dye was removed before loading the sample. To remove any excess ammonium persulfate, current was applied to the tubes at 1 milliamp per tube for 45 minutes. The electrode buffer used consisted of 8.7×10^{-2} M Tris, 8.8×10^{-3} M Tris, 8.8×10^{-3} M boric acid and 2.5×10^{-3} M disodium ethylenediamine tetraacetate (EDTA) at pH 8.9.

Ten flies were hand homogenized in 1 ml of the Tris-EDTA-Borate buffer with 1 M sucrose added in a 2 ml Ten Broeck (Kontes) glass grinder. After homogenization, 25 µl was loaded on top of the gels using Drummond disposable micropipettes.

Electrophoresis was carried out at 2 milliamps per tube (Buchler Constant Current Power Supply) until the tracking dye neared the end of the tube (about 2 hours). Temperature was maintained at 0 to 5 C. The gels were removed from glass tubes using water pressure and a cannula. The gels were then immersed in 50 ml of 0.2 M Tris (pH 8.0) 10^{-3} M hypoxanthine, 3.9 x 10^{-4} M tetranitro blue tetrozolium, 2.6 x 10^{-4} M phenazine methosulfate and 4.5 x 10^{-4} M nicotinamide adenine dinucleotide. Violet formazan bands showing the location of XDH could be seen after 2 to 3 hours at 25 C.

Gel Electrofocusing

The apparatus used for disc electrophoresis is also suitable for disc electrofocusing. The same concentrations and techniques used in making the gels for electrophoresis were used for electrofocusing. After polymerization, ammonium persulfate was removed from the gels by passing 1 milliamp current through them for 45 minutes. This procedure was carried out using a cathode solution of 2.5 percent ethylene diamine and an anode solution of 2.5 percent phosphoric acid.

Ampholine, a carrier ampholyte produced by LKB Laboratories, was used to establish a pH gradient in the gel tubes. The carrier ampholyte solution contained 0.05 ml ampholine (pH 3-10) in 2 ml of a 5 percent sucrose solution. The same electrode solution used for removing ammonium persulfate was added carefully to avoid disturbing the ampholyte layer. Current, 1 milliamp per tube,

was applied for 30 minutes to set up a pH gradient. The sample was then layered on top of the gel, under the ampholyte layer, using a Drummond disposable micropipette. The sample was prepared as for electrophoresis. Electrofocusing was performed at 0-5 C at 1 milliamp per tube for 3 hours. The current was gradually increased until the desired maximum current was reached. This current was maintained until the end of the run. Gels were removed and stained in a staining solution as for electrophoresis.

Statistical Analysis

The significance of differences at the 1 percent level between means were determined by Student's *t* test. Analysis of variance of a linear combination was carried out using the method of Ostle (1963, p. 80).

RESULTS

To determine if relocating a gene affects its activity, it was necessary to determine the enzyme activity specified by a normally positioned gene. Therefore, genotypes with 0, 1 or 2 $\underline{ry}^+$ genes present were assayed for xanthine dehydrogenase activity. The data in Table 2, the results of assays on 24 ± 3 hours old imagoes, demonstrate that differences in the dosage of $\underline{ry}^+$ affect XDH activity. Flies homozygous for rosy or heterozygous for rosy and Df(3)$\underline{ry}^{27}$ had no detectable XDH activity. By crossing wild type flies to the rosy mutants, Df(3)$\underline{ry}^{27}$ and $\underline{ry}$, heterozygous flies were produced with a single dose of $\underline{ry}^+$. These flies were phenotypically wild type and visually indistinguishable from flies homozygous for $\underline{ry}^+$. The activity of homozygous two-dose flies was 50, while activities for heterozygous one-dose flies, $\underline{ry}^+/\underline{ry}$ and $\underline{ry}^+$/Df were 25 and 23 respectively. Dosage of the $\underline{ry}^+$ locus is additive, and one dose is enough to produce a phenotypically normal fly. These results are consistent with the work of Glassman (1962a) and Grell (1962).

Knowing the characteristics of normally positioned $\underline{ry}^+$ genes, it was possible to determine if relocating the gene affects its activity. The first stock tested was T(3;4)86D, a rearrangement involving a reciprocal translocation between the right arm of chromosome 3 and the basal portion of the right arm of chromosome 4. According to Grell (1959a, 1959b) salivary gland chromosome analysis by Lewis shows breaks in the third chromosome at 86D1-2 and in chromosome 4 at

Table 2. Xanthine dehydrogenase activity in genotypes with normally positioned rosy alleles. Enzyme activities expressed as nm isoxanthopterin produced /mg protein/minute.

Genotypes	Sex	Doses of $\underline{ry}^{+}$	Activity	Std. error	No. of Experiments
Canton-s	Mix	2	50	0.6	10
$\underline{ry}^{+}/\underline{ry}$	Mix	1	25	0.4	10
$\underline{ry}^{+}$/Df	Mix	1	23	0.8	5
$\underline{ry}$ /$\underline{ry}$	Mix	0	0.1	0.08	10
$\underline{ry}$/Df	Mix	0	-0.02	0.04	5

101 F. The $\underline{ry}^+$ gene carried on the T(3;4)86D chromosome will be referred to as T($\underline{ry}^+$) for convenience.

A number of crosses were made to study the activity of T($\underline{ry}^+$). Table 3 lists the results of assays of 24 ± 3 hours old adult flies containing relocated $\underline{ry}^+$ genes. It is clear that T($\underline{ry}^+$)/T($\underline{ry}^+$) flies had a much higher level of activity (73) than the control Canton-s (50) and that this difference is significant at the 1 percent level. To determine if the high level of activity was due to the $\underline{ry}^+$ allele present in T($\underline{ry}^+$)/T($\underline{ry}^+$), the heterozygotes T($\underline{ry}^+$)/$\underline{ry}^+$ and T($\underline{ry}^+$)/$\underline{ry}$ were made and tested. Their activities were 49 and 26 respectively. These latter values are not significantly different from the controls at the 1 percent level.

Keller and Glassman (1964b) have reported some variation in XDH activity between strains. They also reported an increased enzyme activity in the heterozygotes between two strains. Their results were reported as activities per number of flies. To be sure that the increased activity detected in homozygous T($\underline{ry}^+$) genotypes was real, activities per number of flies were determined (Table 3). It is clear that the difference in activity between homozygous T($\underline{ry}^+$) and control genotypes is even greater when the denominator is number of flies. The heterozygote T($\underline{ry}^+$)/$\underline{ry}^+$ also showed an increase in activity relative to the control when number of flies were used as a denominator. There was no significant increase in activity in the heterozygote T($\underline{ry}^+$)/$\underline{ry}^+$ when the denominator was milligrams of protein. This points out the importance of using a denominator such as soluble protein when comparing activities between two

Table 3. Xanthine dehydrogenase activity in genotypes with relocated rosy genes and a comparison of Canton-s males and females. Enzyme activities expressed as nm isoxanthopterin produced /mg protein/minute and change in flurometric units/fly.

Genotypes	Sex	Doses of $\underline{ry}^+$	Activity	Std. error	F.U./fly[a]	# Experiments
$T(\underline{ry}^+)/T(\underline{ry}^+)$	Mix	2	73	0.3	32	10
$T(\underline{ry}^+)/\underline{ry}^+$	Mix	2	49	0.5	21	10
$T(\underline{ry}^+)/\underline{ry}$	Mix	1	26	0.5	13	10
Canton-s	Males	2	51	0.6	18	8
Canton-s	Females	2	53	0.6	21	8

[a]Flurometric units/per fly.

strains. To act as a further control on any physiological variation in strains, G6PD activity in all of the above strains was determined using the same homogenate as used for the XDH assay. Using soluble protein as a denominator there was no difference significant at the 1 percent level between any of these strains and the control.

To determine if increased activity is a common phenomena associated with the relocation of $\underline{ry}^{+}$ genes, a second rearrangement was examined. This stock, Df(3)$\underline{ry}^{81}$/Df(3)$\underline{ry}^{81}$/Dp(3;4)$\underline{ry}^{+}$ contains a duplication of the rosy locus. Grell (1962) produced this duplication by irradiating males heterozygous for T(3;4)86D and $\underline{st}\ \underline{ry}^{2}\ \underline{sr}\ \underline{e}^{s}$ with 4000 r. These irradiated males were mated to homozygous $\underline{st}\ \underline{ry}^{2}\ \underline{sr}\ \underline{e}^{s}$ virgin females. A $\underline{st}\ \underline{ry}^{+}\ \underline{sr}\ \underline{e}^{s}$ male was found among the progeny. Tests indicated that this fly carries a small centric fragment (88B-86D3/101F-101D) containing $\underline{cu}^{+}$, $\underline{kar}^{+}$ and $\underline{ry}^{+}$. Chovnick (personal communication) introduced this Dp(3;4)$\underline{ry}^{+}$ into the stock Df(3)$\underline{ry}^{81}$/Df(3)$\underline{ry}^{81}$ to facilitate stock keeping. For convenience this stock will be referred to as Dp($\underline{ry}^{+}$). Comparison of Dp($\underline{ry}^{+}$) activity levels with controls shows significant increases in levels of XDH activity (Table 4).

Activity levels were not significantly different from controls in stocks containing a relocated $\underline{ry}^{+}$ and a structurally normal third chromosome. Examples of this are T($\underline{ry}^{+}$)/$\underline{ry}^{+}$ (49) compared with Canton-s (50) and T($\underline{ry}^{+}$)/$\underline{ry}$ (26) compared to $\underline{ry}^{+}$/$\underline{ry}$ (25).

Table 4. Xanthine dehydrogenase activity in Dp(3;4)$\underline{ry}^{+}$ and control. Enzyme activities expressed as nm isoxanthopterin produced /mg protein/minute.

Genotype	Sex	Doses of $\underline{ry}^{+}$	Activity	Std. error	No. of experiments
Dp(3;4)$\underline{ry}^{+}$	Mix	1	33	0.6	10
$\underline{ry}^{+}/\underline{ry}$	Mix	1	25	0.4	10

Activity is significantly higher in stocks which contain a relocated $\underline{ry}^+$ gene and lack a structurally normal third chromosome. Examples of this are T($\underline{ry}^+$)/T($\underline{ry}^+$) (73) compared with Canton-s (50) and Df(3)$\underline{ry}^{81}$/Df(3)$\underline{ry}^{81}$/Dp(3;4)$\underline{ry}^+$ (33) compared with $\underline{ry}^+/\underline{ry}$ (25). The data presented to this point indicates that there is some property of structurally normal third chromosomes that is influencing xanthine dehydrogenase activity. According to the data on Df(3)$\underline{ry}^{81}$/Df(3)$\underline{ry}^{81}$/Dp(3;4)$\underline{ry}^+$, it would appear that this property of the structurally normal third chromosome is located in the region of the deficiency Df(3)$\underline{ry}^{81}$.

The increased activity of the relocated $\underline{ry}^+$ genes could be explained by the following possibilities: 1) the increased activities are the result of a change in the structural genes, thus producing different isoalleles having different enzyme activities; 2) increased activities are the result of a change in a control factor affecting the rosy locus or 3) the developmental profile could be shifted.

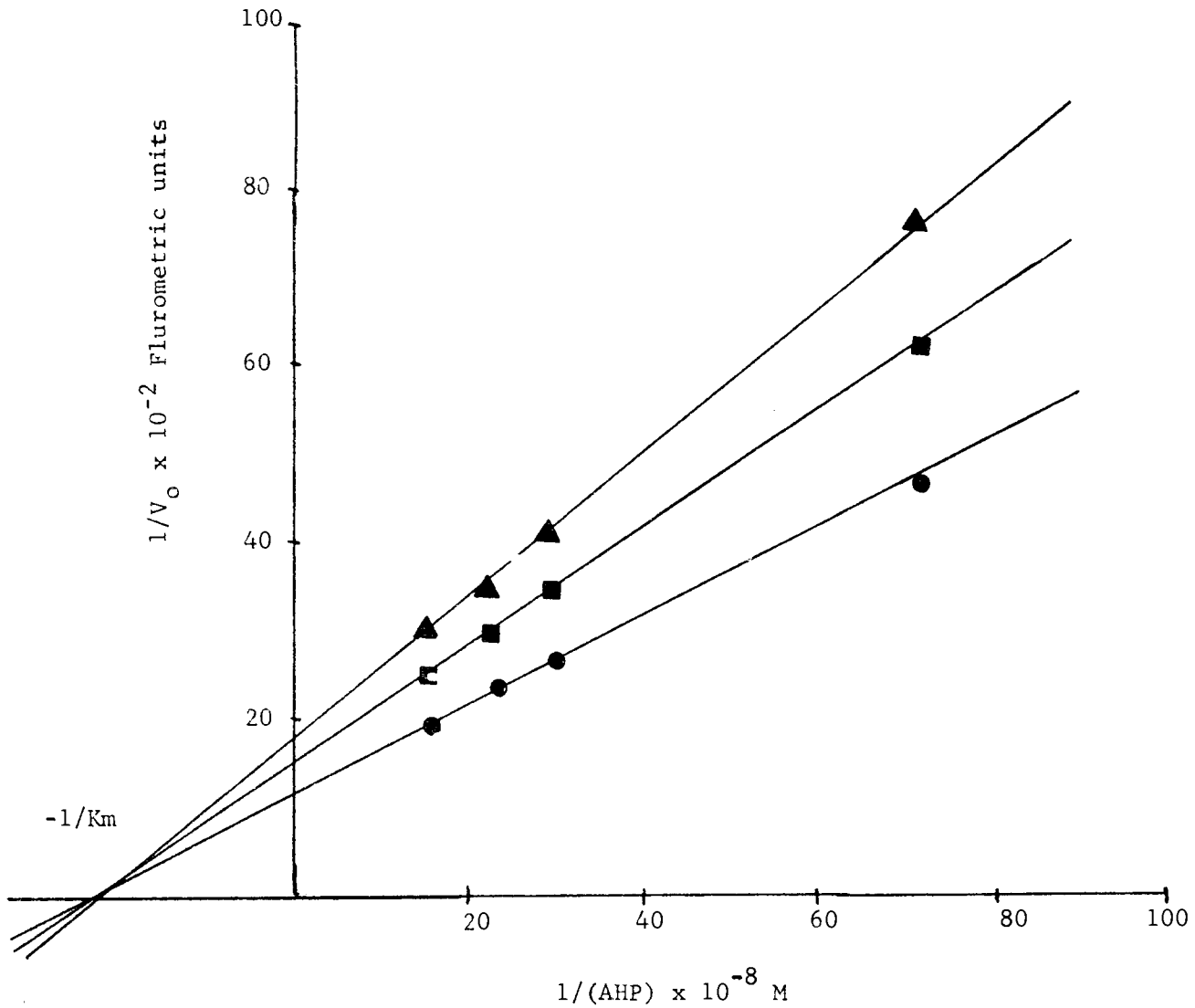
To test the first possibility, the relocated $\underline{ry}^+$ genotypes T($\underline{ry}^+$) and Dp($\underline{ry}^+$) were tested to determine if the XDH enzymes produced by these genotypes are electrophoretically separable. Enzyme produced by T($\underline{ry}^+$) and Dp (ry^+) shows no difference in electrophoretic mobility from that of the control Canton-s, but XDH enzyme produced by all three genotypes, T($\underline{ry}^+$), Dp($\underline{ry}^+$) and Canton-s have a different electrophoretic mobility than the enzyme from D. virilis.

With electrofocusing there is no separation between enzymes produced by relocated genes and Canton-s, but they were separable from XDH enzyme produced by D. virilis. Electrofocusing does give more clearly defined XDH bands and better separation than electrophoresis.

Homogenates containing XDH activity were prepared from relocated and control genotypes, T(ry^+), Dp(ry^+) and Canton-s respectively, and the kinetic properties studied. The Km of 2-amino-4-hydroxypteridine (AHP) with methylene blue as the electron acceptor was 4.4 μM from T(ry^+), 4.5 μM for Dp(ry^+) and 4.9 μM for Canton-s at pH 7.2 (Figure 3). These values for the Km of AHP are not significantly different at the 1 percent level and agree with values previously reported (Glassman and Mitchell, 1959a; Yen and Glassman, 1967).

Preparations of XDH enzyme produced by relocated and control genotypes were incubated for 5 minutes at temperatures from 20 to 80 C at 5 C intervals (Figure 4). No loss of XDH activity was observed for either the extracts from the control or relocated genomes when incubation was from 20 to 40 C. Incubation at 40 to 70 C resulted in a decrease in activity as temperature was increased. The relative decrease in activity was the same in xanthine dehydrogenase produced by relocated and normally positioned genes. From 70 to 80 C there was a sharp decrease in XDH activity in both relocated and control genotypes. There appeared to be no difference in the response to temperature between XDH enzyme produced by relocated and control genotypes.

Figure 3. Lineweaver-Burke plot determining the Km of 2-amino-4-hydroxypterine (AHP) with xanthine dehydrogenase prepared from Canton-s (▼), T($\underline{ry}^+$)/T($\underline{ry}^+$) (●), and Dp($\underline{ry}^+$) (■) genotypes (pH 7.2). Each plot represents the least squares line for five separate assays.


100
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$1/V_o \times 10^{-2}$ Flurometric units
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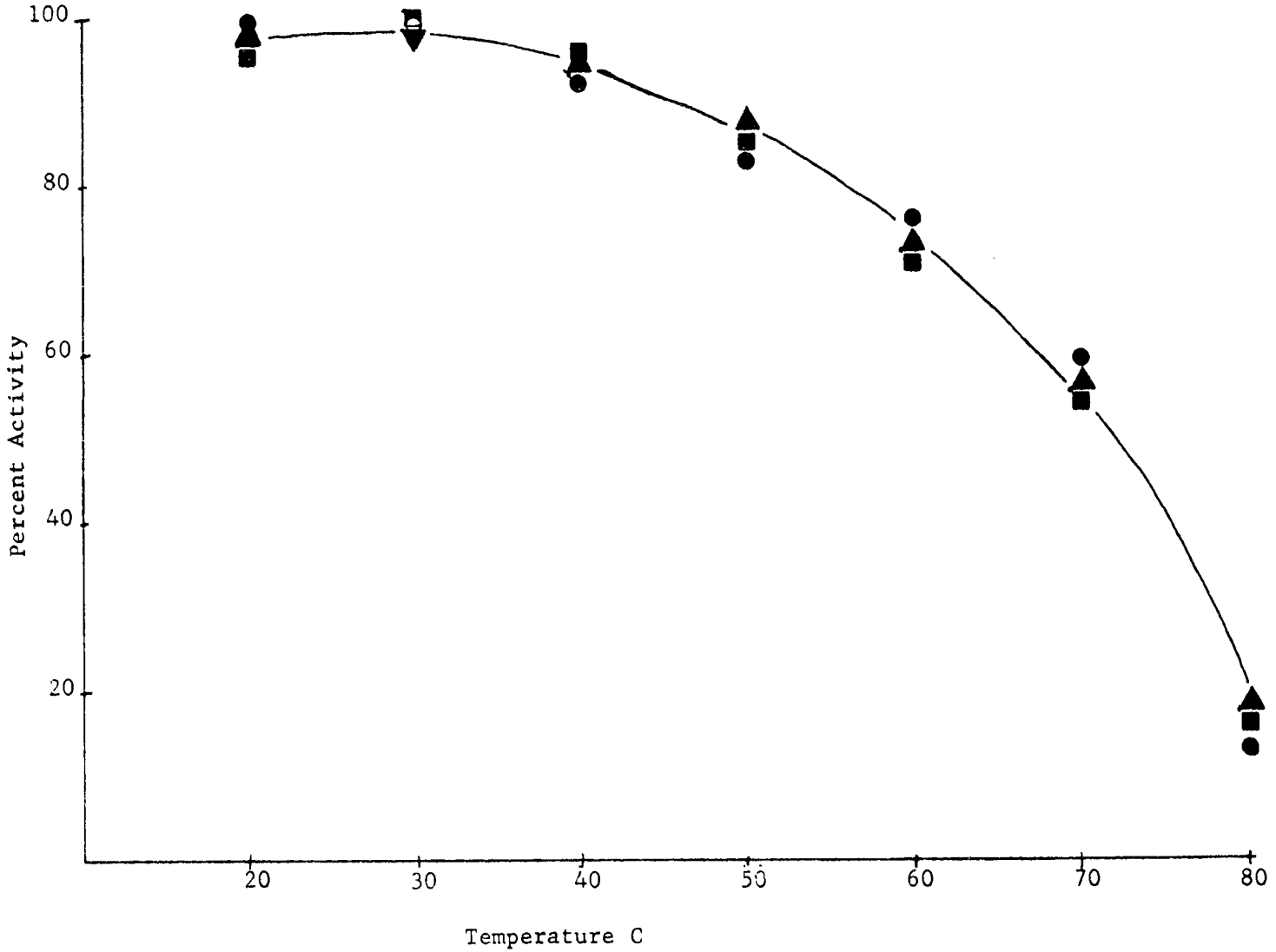
80
100
$1/(AHP) \times 10^{-8}$ M

Figure 4. Temperature inactivation curve of Canton-s (▼), T($\underline{ry}^+$)/T(ry^+) (●), and Dp($\underline{ry}^+$) (■) genotypes. The abscissa is temperature in degrees centigrade and the ordinate is percent activity.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Percent Activity
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The results obtained with flies 24 ± 3 hours old indicated that there was a difference in activity between relocated and normally positioned $\underline{ry}^+$ genes. Therefore, the following experiments were performed to determine if gene activity during ontogeny is influenced by the genes chromosomal position. The relocated genes found in T($\underline{ry}^+$) and Dp($\underline{ry}^+$) were used for the following experiments. Ontogenic profiles of XDH and G6PD activities were determined for relocated $\underline{ry}^+$ genotypes and normally positioned $\underline{ry}^+$ genotypes. G6PD was used as a control of general physiological conditions of the genotypes tested and to check synchrony of developing flies in these different genotypes.

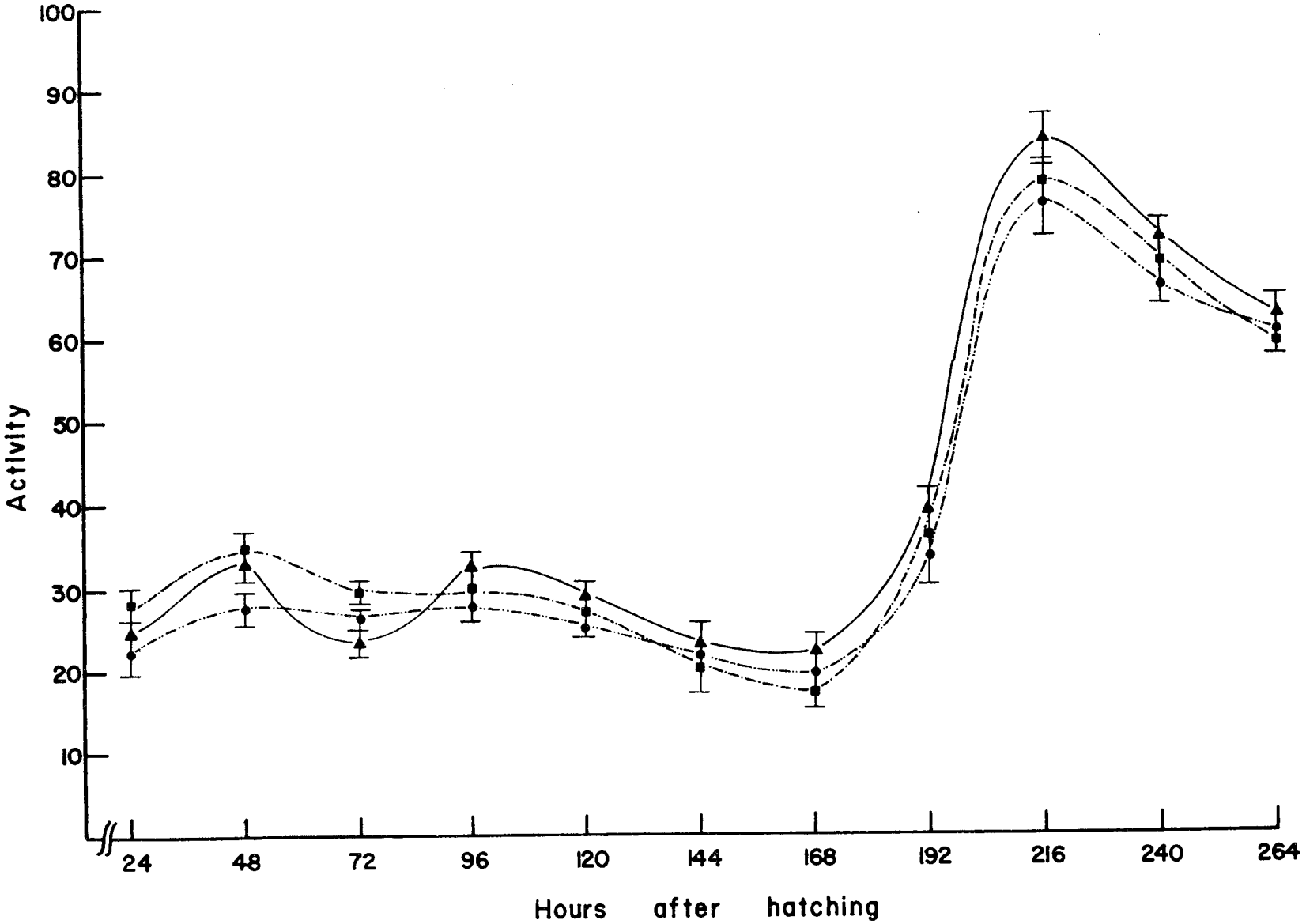
Results showing the G6PD activity profiles are given in Figure 5. Larval stages include samples collected up to 96 hours after hatching; 96-100 hour samples were drawn from non-floating pupae. Samples of pupae collected during 100-196 hours after hatching were synchronized by collecting floaters at 100 hours. Larvae and pupae were assayed at 24 hour intervals until eclosion. Adults eclose at approximately 196 hours after hatching. There was no significant difference in the profiles of relocated and control genotypes for G6PD. The relocated and control genotypes appear to be in close developmental synchrony as can be seen by their respective profiles.

The genotypes T($\underline{ry}^+$)/$\underline{ry}^+$, T($\underline{ry}^+$)/$\underline{ry}$ and $\underline{ry}^+$/$\underline{ry}$ were assayed for G6PD activity. The results of these G6PD assays were compared with the G6PD activity profiles for homozygous T($\underline{ry}^+$), Dp($\underline{ry}^+$) and Canton-s, none were found to differ significantly.

Figure 5. Glucose-6-phosphate dehydrogenase activity in Canton-s (▲), T(3;4)86D (●) and Dp(3;4)$\underline{ry}^{+}$ (■) genotypes during ontogeny. Activities are expressed as nm NADP reduced / min / mg protein and represented as the mean and standard error of the mean for more than five assays.

The developmental stages included on the abscissa are:

1) Larvae . . . . . 24-72 hours after hatching
2) Pupae . . . . . 96-172 hours after hatching
3) Adults . . . . . 196-264 hours after hatching


100
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Activity
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168
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240
264
Hours after hatching

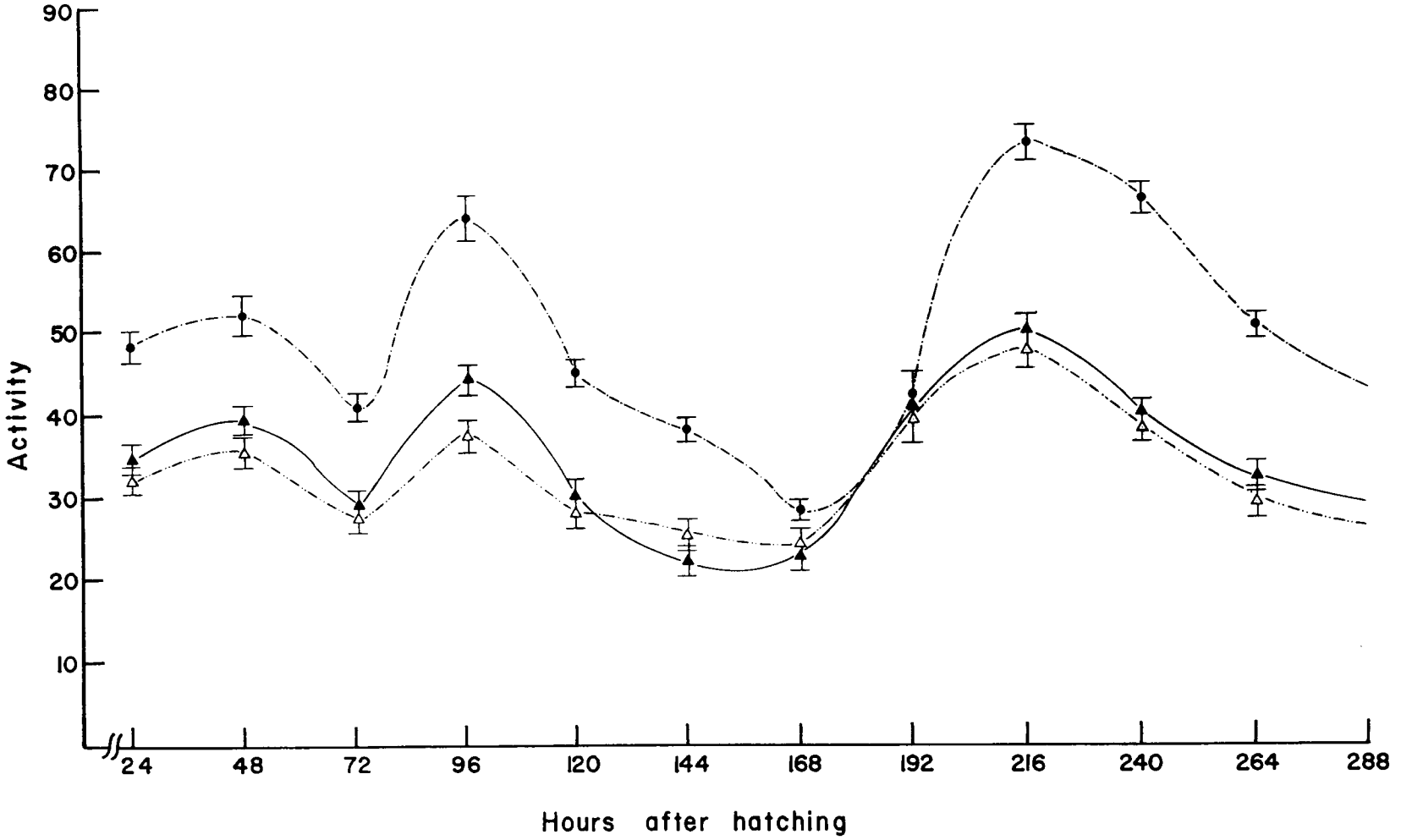
The results of assays on genotypes containing two doses of $\underline{ry}^+$ (homozygous T($\underline{ry}^+$), T($\underline{ry}^+/\underline{ry}^+$ and Canton-s) are given in Figure 6. The activity for homozygous T($\underline{ry}^+$) is significantly higher than the control Canton-s at all periods except 168-192 hour old pupae. The increased activity is more pronounced in 96 hour old pupae and 24 hour old imagoes. The increased activity of these two peaks in T($\underline{ry}^+$) is significantly greater, tested by analysis of variance of a linear combination, than the general increased level of XDH activity in T($\underline{ry}^+$).

The results of assays on genotypes containing one dose of $\underline{ry}^+$(Dp($\underline{ry}^+$) and $\underline{ry}^+/\underline{ry}$) are given in Figure 7. Activity in Dp($\underline{ry}^+$) is significantly higher than the control $\underline{ry}^+/\underline{ry}$ throughout development except for 144-192 hour old pupae. These results are consistent with the results for two doses of $\underline{ry}^+$. As in genotypes with two doses of $\underline{ry}^+$, a single dose of $\underline{ry}^+$ Dp($\underline{ry}^+$) shows a more pronounced increase in activity in 96-hour old larvae and 24-hour old adults. The increased activity of 96-hour old pupae and 24-hour old imagoes in Dp($\underline{ry}^+$) is significantly greater than the general increased level of XDH activity in Dp($\underline{ry}^+$).

Figure 6. Xanthine dehydrogenase activity in Canton-s (▲), T(3;4)86D (●), and T($\underline{ry}^{+}$)/$\underline{ry}^{+}$ (△) genotypes during ontogeny. Activities are expressed as nm isoxanthopterin produced / mg protein / minute, and represented as the mean and standard error of the mean for more than five assays.

The developmental stages included on the abscissa are:

1) Larvae . . . . . 24-72 hours after hatching
2) Pupae . . . . . 96-172 hours after hatching
3) Adult . . . . . 196-264 hours after hatching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Activity
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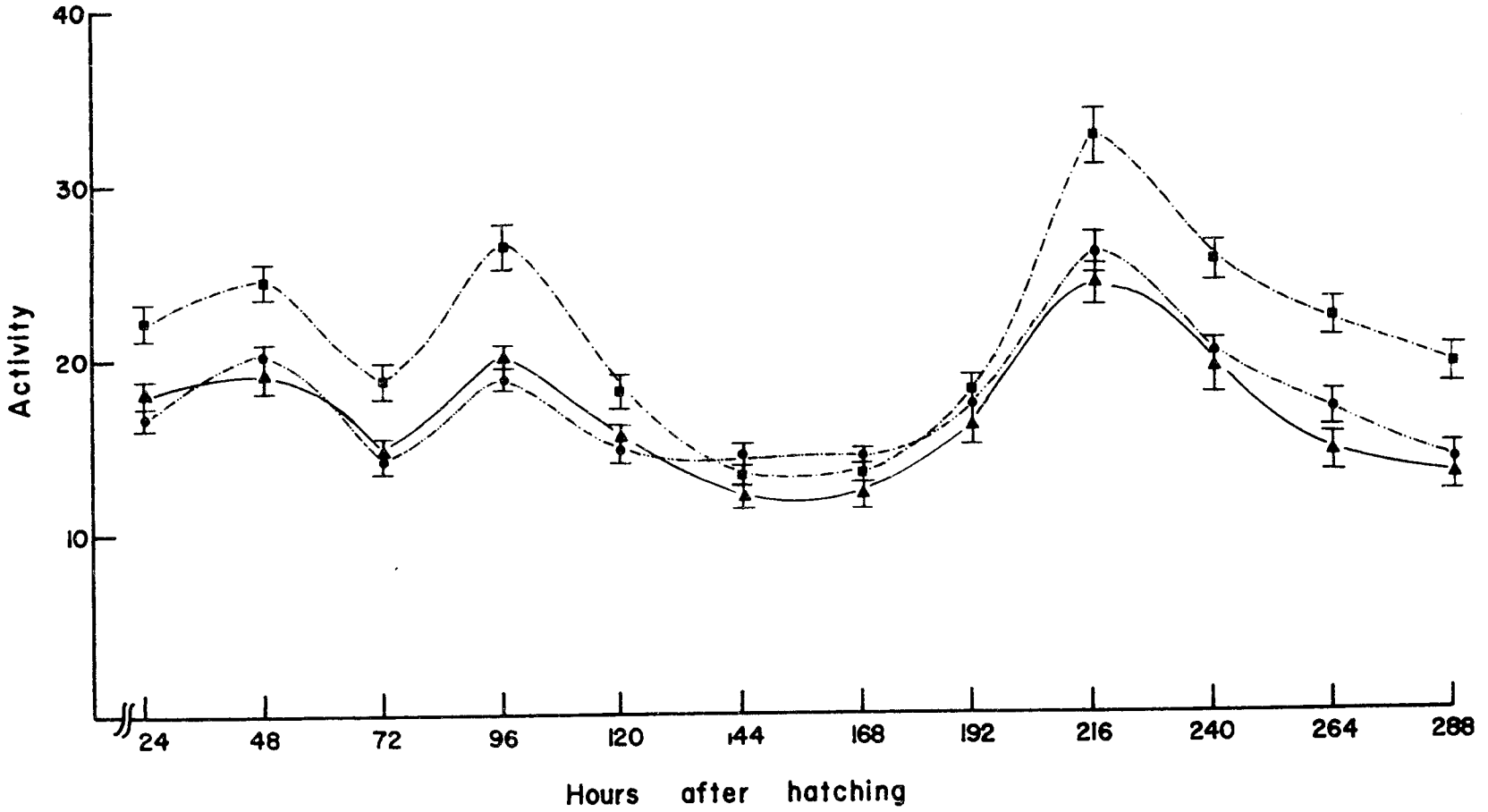
264
288
Hours after hatching

Figure 7. Xanthine dehydrogenase activity in $\underline{ry}^{+}/\underline{ry}$ (▲), $T(\underline{ry}^{+})/\underline{ry}$ (●) and $Dp(\underline{ry}^{+})$ (■) genotypes during ontogeny. Activities are expressed as nm isoxanthopterin produced / mg protein / minute, and represented as the mean and standard error of the mean for more than five assays.

The developmental stages included on the abscissa are:

1) Larvae . . . . . 24-72 hours after hatching
2) Pupae . . . . . 96-172 hours after hatching
3) Adults . . . . . 196-264 hours after hatching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Hours after hatching

DISCUSSION

The results of this study on the effect of relocation of the structural gene for xanthine dehydrogenase in Drosophila melanogaster can be summarized by the following statements: 1) the gene is differentially active during ontogeny; 2) xanthine dehydrogenase activity during ontogeny is proportional to the number of ry^+ genes present in the genome; 3) there is no difference in the electrophoretic mobility or kinetic parameters of xanthine dehydrogenase produced by relocated ry^+ genes, compared to that produced by Canton-s; and 4) a unique level of xanthine dehydrogenase is associated with relocated ry^+ genes in two genotypes tested which lack a structurally normal third chromosome.

The profile of xanthine dehydrogenase activity determined in this study is in general agreement with the data of Ursprung (1961) and Munz (1964). These workers determined xanthine dehydrogenase activity in third instar larvae, early pupae, and in imagoes of various ages. Ursprung reported an increase in activity with age of imagoes. This conflicts with the results found in the present study and those reported by Munz (1964). The data on third instar larvae and early pupae reported by Ursprung (1961) and Munz (1964) are in agreement with the results found in the present study.

There is no difference in activity between adult males and females. This is contrary to results reported by Glassman (1962a) but is consistent with the results reported by Grell (1962) and Parzen and Fox (1964).

The profile of xanthine dehydrogenase activity during development indicates that $\underline{ry}^+$, the structural gene for xanthine dehydrogenase, is differentially active during ontogeny. These results are consistent with the ontogenic profiles of a number of enzymes, for example, alcohol dehydrogenase (Courtright, 1967), G6PD and 6PGD (Bowman and Simmons, In press) and tryptophan pyrrolase (Tobler, 1971). This differential activity during development is evidence of a temporal regulation of protein synthesis.

A comparison of the xanthine dehydrogenase profiles of genotypes containing zero, one and two doses of $\underline{ry}^+$ clearly shows a dosage effect throughout development. These results are consistent with dosage effect in adults as reported by Glassman (1962b) and Grell (1962, 1969). Bowman and Simmons (In press) have reported a similar response to dose of the sex linked $\underline{Pgd}^+$ and $\underline{Zw}^+$ genes in pupae and adults of Drosophila melanogaster. The fact that $\underline{ry}^+$ shows dosage additivity throughout development indicates a regulatory function acting on $\underline{ry}^+$ that operates on the individual gene or perhaps a restricted chromosomal segment. Control functions such as feedback inhibition and substrate induction seem to be lacking since activity is dependent primarily on the number of $\underline{ry}^+$ genes in the genome.

According to a number of criteria, there is no difference between the xanthine dehydrogenase produced by relocated $\underline{ry}^+$ genes and that produced in Canton-s. Determination of electrophoretic mobility, Km and temperature inactivation profiles failed to reveal any variant form of xanthine dehydrogenase

produced by relocated $\underline{ry}^+$ genes. Multiple molecular forms of xanthine dehydrogenase have been reported by Collins, Duke and Glassman (1971). Such forms do not seem to explain the results obtained in this work.

The fact that $\underline{ry}^+$ is differentially active during ontogeny and that dosage additivity is present throughout development is not surprising. These results are consistent with the work done on a number of enzymes. The results showing that the physical properties of xanthine dehydrogenase were not changed due to a relocation of the $\underline{ry}^+$ gene were also expected. The unique level of xanthine dehydrogenase associated with relocated $\underline{ry}^+$ genes in several genotypes which lack a structurally normal third chromosome was not expected.

The profiles of xanthine dehydrogenase activity in genotypes with a relocated $\underline{ry}^+$ shows that there is an increase in activity in $T(\underline{ry}^+)/T(\underline{ry}^+)$ and Dp $(\underline{ry}^+)$. There is no increase in activity in the genotypes $T(\underline{ry}^+)/\underline{ry}^+$ and $T(\underline{ry}^+)/\underline{ry}$. One might expect all genotypes containing a relocated $\underline{ry}^+$ gene to either show or not show a change in activity. Upon close inspection, it is clear that the genotypes with a relocated $\underline{ry}^+$ gene and a structurally normal third chromosome fail to show any increased XDH activity. Those genotypes with relocated $\underline{ry}^+$ genes and lacking a structurally normal third chromosome have an increased level of xanthine dehydrogenase activity.

The reason for the lack of increased activity in genotypes containing relocated $\underline{ry}^+$ genes and structurally normal third chromosomes is not clear. As far as this author can determine, no similar effect has been reported in the literature.

The increase in activity of T($\underline{ry}^+$)/T($\underline{ry}^+$) and Dp($\underline{ry}^+$) is present throughout development except in the late pupal stage. This decrease in XDH activity during the late pupal stage is similar to previously reported low enzyme levels at this stage in a number of enzymes, examples are: tryptophan pyrrolase activity (Tobler, 1971) and G6PD (Bowman and Simmons, In press). This may be a reflection of a general decrease in activity during the late pupal stage since it is characteristic of all three of the above enzymes.

Two possible precedents for the modification of gene activities associated with gene relocation are: 1) position effect; and 2) isoalleles, a change in the allele causing a change in enzyme structure with a resultant change in enzyme activity.

The relocation of a gene from a euchromatic region into the vicinity of heterochromatin is often accompanied by an alteration in that gene's expression (Baker, 1968, for review). The effects of these chromosomal rearrangements are referred to as variegated position effects. Variegated position effects have the property of affecting several genes in the vicinity of the heterochromatic breakpoint.

The variegated effect shows polarization since inactivation spreads from the heterochromatic break to the nearest gene first (Demerec and Slizynska, 1937 and Demerec, 1940). The suppressing effect spreads along the chromosome affecting the genes most proximal to the break point first.

The rearrangements involved in the present study on the effect of relocation have the $\underline{ry}^+$ gene relocated into the vicinity of heterochromatin.

The ry^+ gene when relocated is at the known limits of the polarized spreading effect. Although the requirements for variegated position effect have been met, it is unlikely that a position effect is acting directly on the ry^+ gene, since the ry^+ gene is so distant from the heterochromatic breakpoint. Also variegated position effects, as a general rule, decrease gene activity rather than increase it.

Dunn, Wilson and Jacobson (1960) have reported that isozymes of alcohol dehydrogenase have different associated activity levels. They report a change in relative concentrations of isozymes during development in Drosophila.

The available data on the physical characteristics of xanthine dehydrogenase do not eliminate the possibility that relocation of ry^+ genes results in the formation of an isoallele with altered enzyme activity. The data do make this possibility seem unlikely.

Recently Britten and Davidson (1969) have proposed a model to explain regulation of gene activity in higher cells. An underlying principle in their model is that the producer gene (structural gene) need not be physically linked to its control elements, regulator genes. Their model proposes that there are a sizable number of regulator genes present in the genome. As evidence for the presence of regulator genes in Drosophila, they cite the example of "the effect of the notch genes" as being consistent with what is expected of regulator genes. They assume that producer genes are activated by the annealing of specific RNA, produced by regulator genes, to receptor genes. The receptor genes are physically linked to the producer genes.

The Britten and Davidson Model is in agreement with much of what we now know of $\underline{ry}^+$ regulation. It is postulated that the $\underline{ry}^+$ gene is a producer gene under the control of a regulator gene or genes. Normally at least one important regulator gene is in proximity to the $\underline{ry}^+$ gene. In genotypes with the $\underline{ry}^+$ gene relocated, the regulator gene is removed from the immediate vicinity of the rosy locus. The regulator gene produces a diffusible substance that activates or represses the $\underline{ry}^+$ gene. To be consistent with the data presented here, the regulator gene would be located to the left of the rosy locus between bands 86D1-2 and 87D8-12. Considering the results obtained with the genotype Dp($\underline{ry}^+$), it seems likely that the regulator gene is located between 87C2 and 87D8-12.

The model presented here specifically proposes that relocation of the $\underline{ry}^+$ gene results in alteration of the activity of an associated regulator gene. Two possible explanations for the effect on the regulator gene are: 1) a structural change in or deletion of the regulator gene resulting in inactivation; and 2) a position effect on the regulator gene resulting in inactivation. The present data fail to distinguish between these two possibilities.

Both of the above possibilities are consistent with the observation that the presence of a structurally normal chromosome in the genotypes tested resulted in normal levels of xanthine dehydrogenase. The structurally normal third chromosome insures the presence of a regulator gene unaffected by relocation. This regulator gene produces a sufficient amount of the diffusible

control substance to activate or repress the $\underline{ry}^+$ genes present in the genome.

The available data do not explain the molecular events involved in $\underline{ry}^+$ regulation and any proposed model must be speculative. They do indicate that the control functions need not be contiguous on the chromosome. The apparent propinquity of the regulator gene to the rosy locus may suggest a form of segmental control of the chromosome.

SUMMARY

The $\underline{ry}^+$ gene of Drosophila melanogaster and its associated enzyme, xanthine dehydrogenase, were employed in an analysis of the relationship between regulation and the location of a gene in the genome. The gene $\underline{ry}^+$ is differentially active during ontogeny and the xanthine dehydrogenase activity associated with $\underline{ry}^+$ is proportional to the number of $\underline{ry}^+$ genes present in the genome.

According to a number of criteria, there is no difference between xanthine dehydrogenase produced by relocated $\underline{ry}^+$ genes and that produced in Canton-s. Determination of electrophoretic mobility, Km and temperature in activation profiles failed to show a variant form of xanthine dehydrogenase produced by relocated $\underline{ry}^+$ genes.

The profiles of xanthine dehydrogenase activity in relocated and control genotypes show that there is an increase in activity in the relocated genotypes. The increased activity is significantly higher than the general increase in xanthine dehydrogenase activity of relocated genotypes at two periods during development. The genotypes which show an increased level of xanthine dehydrogenase lack a structurally normal third chromosome.

The increased level of activity is not the result of a general physiological effect since the ontogenic profile of G6PD activity is the same in wild type and relocated $\underline{ry}^+$ genotypes.

The available data from this investigation on the relationship between regulation and the location of a gene in the genome gives a good foundation on which to attack other critical problems of the nature of gene regulation such as: are ontogenic patterns of closely linked loci similar; do different genes moved to the same location exhibit similar patterns of activity, and what are the properties of the functional unit of gene regulation.

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